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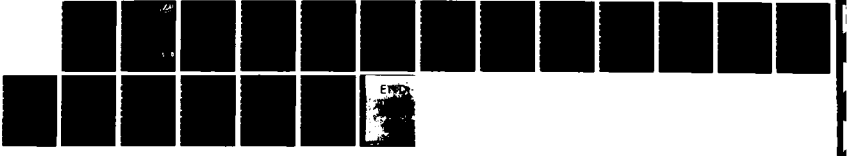

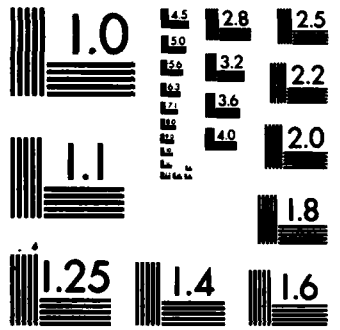

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FREEZE-PRESERVATION OF HORSE RED BLOOD CELLS USING 20% W/V GLYCEROL AND STORAGE AT -150 C FOR 5 YEARS

by

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Horse red blood cells frozen with 20% W/V glycerol and stored at -150 C for as long as 5 years showed no adverse effects on freeze-thaw or freeze-thaw-wash recovery or red cell oxygen transport function related to the length of frozen storage.

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INTRODUCTION

Horse red blood cells were frozen with 20% W/V glycerol and stored in the gas phase of liquid nitrogen at -150 C for up to 5 years. Red cell recovery in vitro, red cell levels of ATP, 2,3 DPG, and P_{50}, and residual hemolysis, were measured in the previously frozen red blood cells on the day of washing. Pre-freeze measurements of red cell ATP, 2,3 DPG, and P_{50} also were made.

METHODS

Each of 5 horses was bled from 2 to 8 separate units of blood during a single day. Four of the 5 horses were bled a second time 6 months after the first phlebotomy. Each 450 ml volume of blood was collected in the primary bag of a double plastic bag system containing 63 ml of citrate-phosphate-dextrose (CPD, Fenwal Laboratories, Deerfield, IL). The blood was stored at 4 C for 1 day.

Just prior to glycerolization and freezing, a sample was taken from each unit of blood for measurements of red cell ATP, 2,3 DPG, and P_{50} levels. Then the blood container was covered with plastic wrap and was placed in a water bath maintained at 37 C for 30 minutes of incubation to increase the temperature of the blood from 4 C to about 22 C. The warmed blood was centrifuged for 7 minutes at 3700 rpm (3500 X g) in a Sorval RC-3 refrigerated centrifuge (DuPont Instruments, Newtown, CT) maintained at 22 $\pm$ 2 C. An empty 300 ml transfer pack integrally attached to the primary bag was used to collect all the visible plasma. Using an AE-7 connector, which is a plastic tube having a male coupler at each end (Fenwal Laboratories, Deerfield, IL), a 30 ml volume of 0.9% sodium chloride solution was added to the red cell concentrate to prevent spontaneous rouleaux formation of the red cells. The red cell mixture was weighed.

The glycerol solution, maintained at 22 C to 30 C and containing per 100 ml: 35.0 g glycerol, 2.88 g mannitol, and 0.065 g sodium chloride (Cytosol Laboratories, Boston, MA), was added in a volume equal to that of the red cell mixture in four separate equal aliquots as follows: The

primary bag containing the red cell mixture was secured to the surface of a modified Eberbach shaker so as to prevent it from moving during addition of the glycerol. The first of the 4 equal aliquots of glycerol solution was added with lateral agitation at 180 cycles per minute over a 5-minute period, and the red cell-glycerol mixture was equilibrated at room temperature for 10 minutes without agitation.

The second aliquot of glycerol was added with agitation, and this was followed by a 10-minute period of equilibration without agitation. The partially glycerolized red cells were transferred from the primary bag to a polyolefin plastic bag (Delmed, Canton, MA). The third and fourth equal volumes of glycerol were added to the polyolefin bag, each addition taking 10 minutes and each followed by a 10-minute period of equilibration without agitation.

The polyolefin plastic bag was placed in an aluminum container, and this container was immersed in liquid nitrogen (-197 C) to promote rapid freezing of the glycerolized red cells. The frozen red cells were stored in the vapor phase of liquid nitrogen at -150 C for up to 5 years.

At various times during the 5-year period, units were selected for evaluation and were thawed by manually agitating the container in a water bath maintained at 42 C for about 6 minutes. The red blood cells were washed as described below.

The wash solution consisted of 500 ml of 3.2% sodium chloride, 2 liters of 1.6% sodium chloride, and 1 liter of 0.9% sodium chloride-0.2% glucose-0.065 g% disodium phosphate (Cytosol Laboratories, Boston, MA),

each maintained at room temperature (22 ± 2 C at the time of use). The 500 ml of 3.2 g% sodium chloride solution was added to the thawed red blood cells at a rate of 40 to 50 ml per minute with gentle manual agitation, after which the mixture was equilibrated for 2 minutes. A 1-liter volume of the 1.6 g% sodium chloride was added to the unit at a flow rate of 100 ml per minute with gentle agitation, followed by equilibration for 2 minutes.

The diluted red cells were recovered in the IBM Blood Processor 2991-1 or 2991-2 (IBM Corp., Princeton, NJ), and were added to the special washing bag of the system in three equal parts. After addition of the first volume, the mixture was sedimented for 2.5 minutes at 2500 rpm. When the centrifuge stopped, the supernatant fluid was decanted at a rate of 450 ml per minute. The rate of pump restoration was set at 300 ml per minute, and the supernatant volume was set at 600 ml. The second and third equal volumes were added to the washing bag with to-and-fro agitation, each addition followed by the process outlined above.

When all the recovered red blood cells were in the washing bag, 500 ml of a 1.6 g% sodium chloride solution was added with to-and-fro agitation at a flow rate of 100 ml per minute. The centrifuge was spun at 2500 rpm for 2 minutes and the supernatant fluid was decanted. The remaining 500 ml of 1.6% sodium chloride solution was added, the red blood cells were concentrated by centrifugation, and the supernatant fluid was removed.

Next, two separate 500 ml volumes of 0.9% sodium chloride solution,

containing 0.2 g% glucose and 0.065 g% disodium phosphate, pH 6.8, were added each at a flow rate of 100 ml per minute with agitation, and each followed by concentration of the red cells by centrifugation and decantation of the supernatant fluid.

The washed red blood cells were resuspended in a 150 ml volume of 0.9% sodium chloride-0.2% glucose-0.065% disodium phosphate solution. The red blood cells, with a hematocrit value of approximately 40 V%, were transferred to a plastic pack and were stored at 4 C for 24 hours before transfusion.

In Vitro Measurements

Red cell ATP, 2,3 DPG and P_{50} were measured in the liquid-stored CPD blood after storage at 4 C for 24 hours and in the previously frozen red blood cells on the day of washing.[4,5] The in vitro recovery of the red blood cells after the freeze-thaw and freeze-thaw-wash procedures was measured as previously described.[2] On the day of washing, measurements were made of red cell mean corpuscular volume (MCV, u^3), mean corpuscular hemoglobin concentration (MCHC, %), mean corpuscular hemoglobin (MCH, uug), supernatant hemoglobin, extracellular potassium and supernatant osmolality (Advanced Instruments, Inc., Needham, MA), red cell 2,3 DPG, ATP, potassium and P_{50}, and blood pH measured at 22 C in a pH/gas analyzer (Radiometer, Copenhagen, Denmark).[3-5]

The Bellingham and Huehns procedure[1] was used to measure the oxyhemoglobin dissociation curve of the washed red cells, and the pO_2 at which 50% of the hemoglobin was saturated at a pCO_2 of 0 and a pH of 7.2, is reported.

RESULTS AND DISCUSSION

Table 1 reports the red cell ATP, 2,3 DPG, and P_{50} values of horse blood after storage at 4 C for 24 hours.

Figure 1 and Table 2 show that there was no correlation between the recovery of the horse red blood cells after the freeze-thaw and freeze-thaw-wash procedures and the length of frozen storage at -150 C. In the 9 studies done in the 5 horses, the mean freeze-thaw recovery value was 96% and the freeze-thaw-wash recovery value was 85%.

The pre-freeze level of red cell 2,3 DPG of 16.4 $\pm$ 2.0 uM/g Hb was significantly higher than the post-thaw-wash level of 14.8 $\pm$ 0.9 uM/g Hb ($t = 3.2$, $p < 0.01$); the pre-freeze level of red cell ATP of 0.78 $\pm$ 0.27 uM/g Hb was significantly higher than the post-thaw-wash level of 0.31 $\pm$ 0.10 uM/g Hb ($t = 6.24$, $p < 0.001$); and the pre-freeze level of red cell P_{50} of 29.6 $\pm$ 2.1 mm Hg was significantly higher than the post-thaw-wash level of 28.1 $\pm$ 1.3 mm Hg ($t = 1.5$, $p < 0.05$) (Tables 1 and 2).

On the day of washing, the supernatant hemoglobin level of 52 mg%, extracellular potassium level of 0.9 mEq/l, and the supernatant osmolality level of 323 mOsm/kg H_2O, showed that minimal hemolysis was present, and the residual glycerol level was less than 1 g%[2] (Table 2).

The 2 to 8 separate units of blood collected from each of 5 horses during a single day, stored at 4 C for 24 hours, and frozen as individual units, were stored at -150 C for up to 5 years to determine how the length of frozen storage influenced the freeze-thaw and freeze-thaw-wash recovery values and the red cell 2,3 DPG, ATP, and P_{50} values.

Our data show that the freeze-thaw and freeze-thaw-wash recovery values and red cell oxygen transport function were not adversely affected by the 5 years of frozen storage at -150 C.

SUMMARY

Horse red blood cells frozen with 20% W/V glycerol and stored at -150 C for as long as 5 years showed no adverse effects on freeze-thaw or freeze-thaw-wash recovery or red cell oxygen transport function related to the length of frozen storage.

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FIGURE 1

The freeze-thaw-wash recovery of horse red cells stored at 4 C for 1 day in citrate-phosphate-dextrose anticoagulant, frozen with 20% W/V glycerol, and stored at -150 C for up to 5 years.

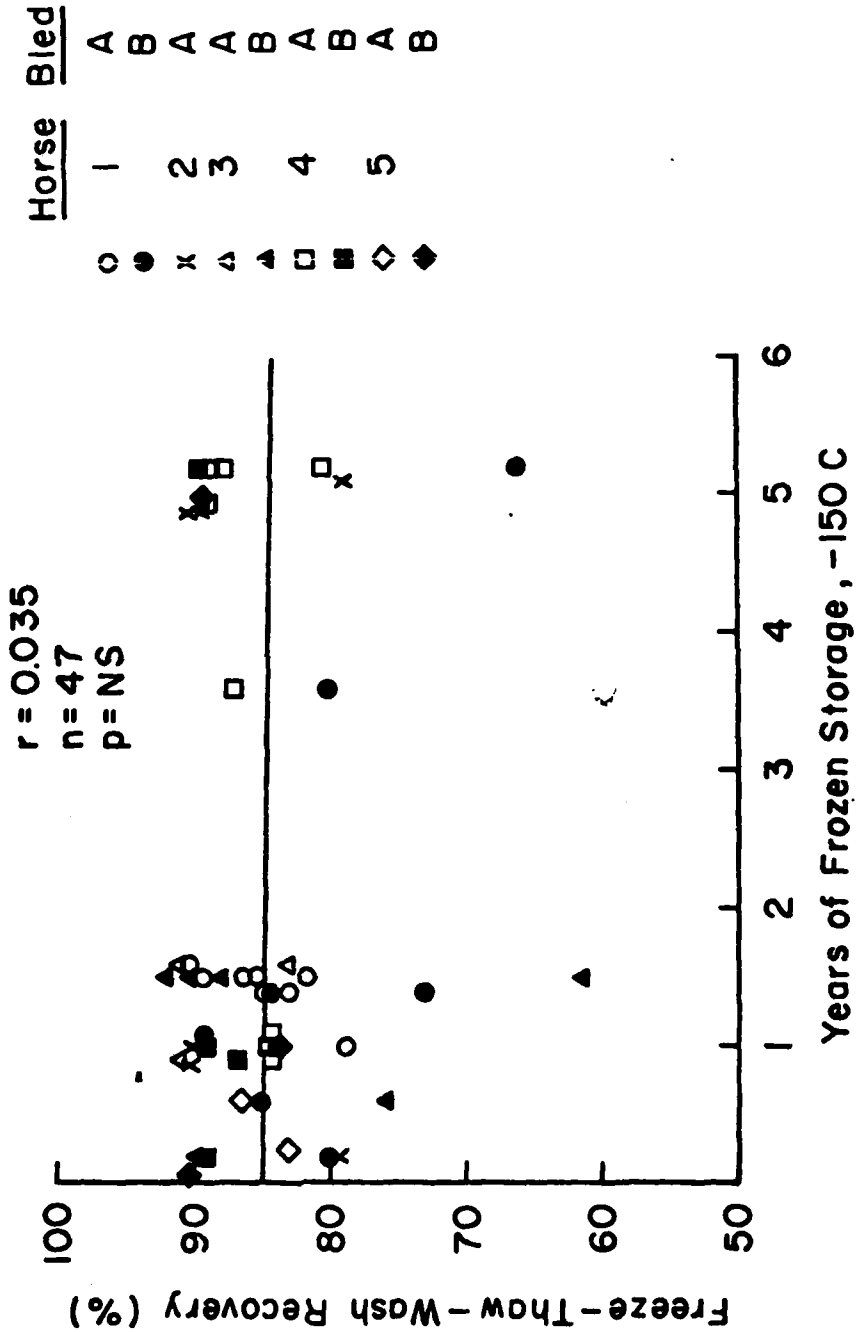

FIGURE 1

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TABLE 1

LEVELS OF RED CELL ATP AND 2,3 DPG AND P_{50} VALUES IN HORSE BLOOD STORED IN CPD AT 4 C FOR 24 HOURS

	ATP (uM/g Hb)	2,3 DPG (uM/g Hb)	P_{50} (mm Hg)
Mean	0.78	16.4	29.6
SD	0.27	2.0	2.1
SE	0.06	0.4	0.5
n	21	21	15

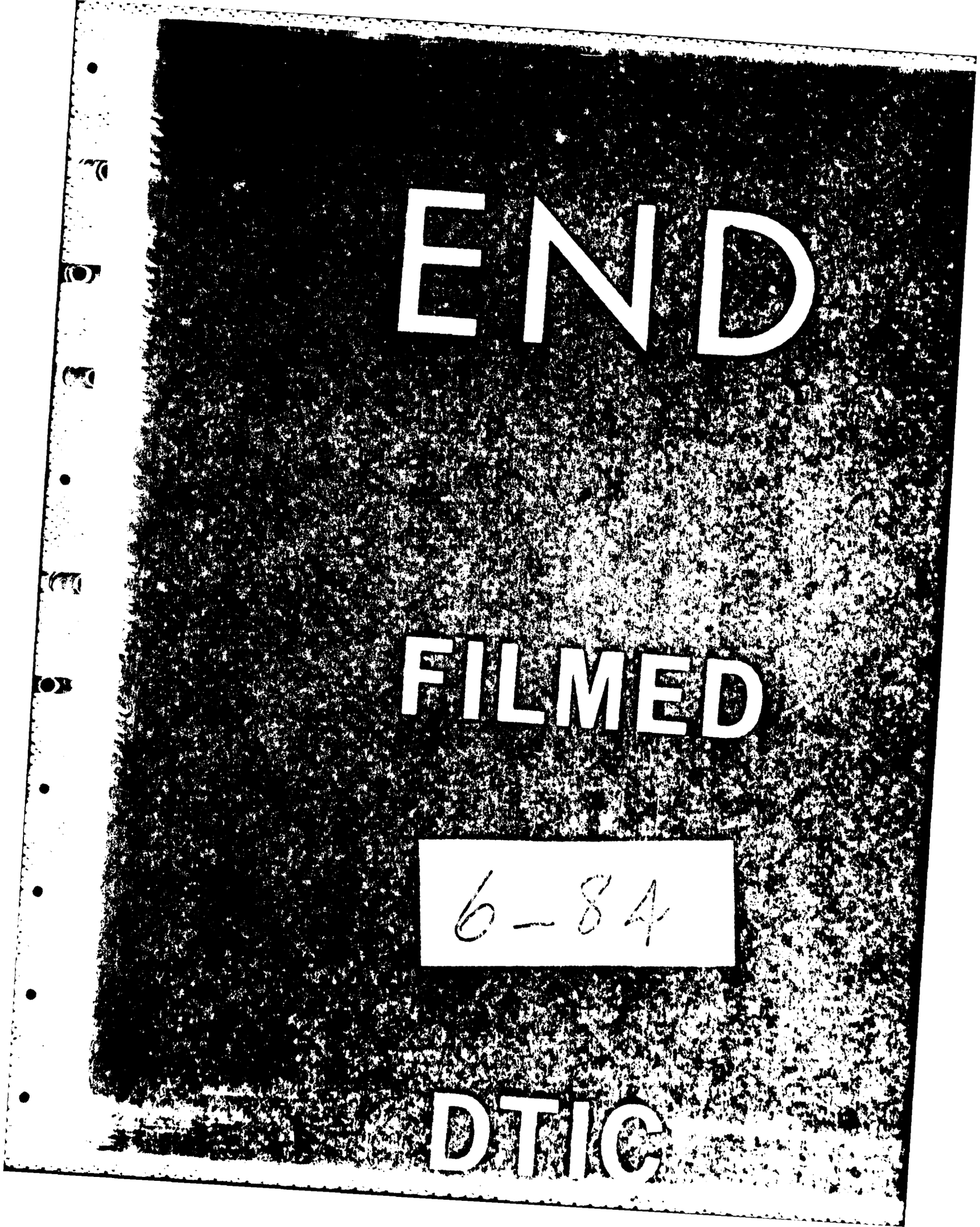
TABLE 2

HORSE RED BLOOD CELLS STORED AT 4 C FOR 1 DAY, FROZEN WITH 20% W/V GLYCEROL AND STORED AT -150 C, AND WASHED IN THE IBM BLOOD PROCESSOR

Horse No.	Bleed	Days Fzn (-150 C)	Recovery (%) Fz	Freeze-Thaw Wash	Supt Hb (mg%)	Extra K^+ (mEq/l)	Supt. Osmolal. (mOsm/kg H_2O)	Blood pH	RBC 2,3 DPG (uM/g Hb)	RBC ATP (uM/g Hb)	RBC P_{50} (mm Hg)	RBC K^+ (mEq/10^{12} RBC)	MCV (u^3)	MCHC (%)	MCH (uug)
1	A	70		79.8	100	0.9	317	6.97	13.7	0.23	29.1	4.2	48.4	38.9	18.8
		227		84.8	48	0.2	327	6.90	----	----	----	---	----	37.8	----
		382	.8	89.2	45	1.8	334	----	----	----	----	4.0	46.8	36.2	16.9
		508	97.1	83.7	65	0.7	324	6.99	14.3	0.12	----	5.3	----	----	----
		508	96.8	73.1	52	0.2	319	6.94	14.4	0.80	----	5.9	----	----	----
		1300	96.7	79.6	122	0.5	410	----	16.2	----	26.3	4.4	48.9	36.3	17.7
		1880	96.9	65.8	34	0.7	303	6.95	18.1	0.35	28.5	4.1	48.4	35.5	17.1
		Mean	96.8	79.4	67	0.7	333	6.95	15.3	0.38	28.0	4.7	48.1	36.9	17.6
		SD	0.4	7.8	32	0.5	35	0.34	1.8	0.30	1.5	0.8	0.9	1.4	0.9
		SE	0.1	3.0	12	0.2	13	0.15	0.8	0.15	0.9	0.3	0.5	0.6	0.4
		n	7	7	7	7	7	5	5	4	3	6	4	5	4
1	B	379	95.9	78.5	48	1.4	320	6.93	----	----	----	----	----	----	----
		510	96.3	83.1	85	3.6	321	7.02	15.2	0.08	----	----	----	----	----
		510	96.0	84.6	77	1.2	307	7.05	15.4	0.12	----	----	----	----	----
		542	96.6	86.2	40	1.1	323	6.95	16.3	0.08	----	----	----	----	----
		542	95.6	85.3	40	0.2	325	7.02	15.3	0.12	----	----	----	----	----
		560	95.4	81.5	30	1.5	300	7.15	13.5	0.18	----	----	----	----	----
		560	96.2	89.3	30	0.5	296	7.12	13.7	0.10	----	----	----	----	----
		Mean	96.0	84.1	50	1.4	313	7.03	14.9	0.11	----	----	----	----	----
		SD	0.4	3.5	22	1.1	12	0.08	1.1	0.04	----	----	----	----	----
		SE	0.2	1.3	8	0.4	5	0.03	0.4	0.02	----	----	----	----	----
		n	7	7	7	7	7	7	6	6	----	----	----	----	----
2	A	66	96.0	79.9	55	3.3	318	6.96	13.4	0.19	28.6	4.0	48.1	38.8	18.7
		307	96.2	90.3	40	0.8	---	7.02	----	----	----	3.8	47.2	35.6	16.8
		371	96.0	90.1	40	1.6	320	----	----	----	----	3.5	43.2	36.4	15.7
		1801	96.1	89.6	110	1.0	---	7.13	----	0.70	23.0	4.6	54.0	39.3	21.1
		1801	96.4	90.1	76	1.0	---	7.12	----	----	----	4.6	54.0	37.8	20.6
		1868	96.6	77.1	41	1.4	299	7.02	16.4	0.20	28.9	4.6	59.0	35.8	21.3
		Mean	96.2	86.1	60	1.5	312	7.05	14.9	0.36	26.8	4.2	50.9	37.3	19.0
		SD	0.2	6.0	28	0.9	12	0.07	2.1	0.29	3.3	0.5	5.8	1.7	2.4
		SE	0.1	2.5	12	0.4	7	0.03	1.5	0.17	1.9	0.2	2.3	0.6	1.0
		n	6	6	6	6	3	5	2	3	3	6	6	6	6

Horse No.	Bleed	Days Fzn (-150 C)	Recovery (%) Fz-Thaw	Recovery (%) Freeze-Thaw-Wash	Supt. Hb (mg%)	Extra K^+ (mEq/l)	Supt. Osmolal. (mOsm/kg H_2O)	Blood pH	RBC 2,3 DPG (uM/g Hb)	RBC ATP (uM/g Hb)	RBC P_{50} (mm Hg)	RBC K^+ (mEq/10^{12} RBC)	MCV (u^3)	MCHC (%)	MCH (uug)
3	A	73	96.1	90.1	50	1.8	314	6.98	13.2	0.24	30.0	3.8	44.7	35.6	15.9
		320	95.9	91.2	20	0.4	---	7.99	----	----	----	4.1	47.3	35.8	16.9
		565	94.9	83.2	20	1.2	---	7.09	13.8	0.28	----	3.5	45.0	33.6	15.0
		565	96.0	90.3	15	0.3	---	7.15	14.3	0.25	----	3.8	44.0	34.9	15.4
		565	96.2	90.3	20	0.7	---	7.15	14.0	0.36	30.5	3.4	40.0	28.9	18.1
		Mean	95.8	89.0	25	0.9	314	7.27	13.8	0.29	30.3	3.7	44.2	33.8	16.3
		SD	0.5	3.2	14	0.6	---	0.41	0.5	0.05	0.4	0.3	2.7	2.9	1.3
		SE	0.2	1.5	6	0.3	---	0.18	0.2	0.03	0.3	0.1	1.2	1.3	0.6
		n	5	5	5	5	1	5	4	4	2	5	5	5	5
3	B	222	98.0	76.2	20	0.6	327	7.05	----	----	----	---	---	38.9	----
		562	96.8	92.3	50	2.2	295	7.14	13.9	0.32	28.5	3.3	42.0	33.3	14.0
		562	96.6	61.4	25	1.7	292	7.31	14.0	0.28	----	3.3	41.0	34.6	14.0
		562	96.4	88.0	32	2.9	293	7.15	14.0	0.15	----	3.0	39.0	33.0	12.8
		562	97.0	90.6	40	0.3	296	7.15	14.4	0.36	----	3.4	41.0	32.5	13.4
		Mean	97.0	81.7	33	1.5	301	7.16	14.1	0.28	28.5	3.3	40.8	34.5	13.6
		SD	0.6	13.0	12	1.1	15	0.09	0.2	0.09	----	0.2	1.2	2.6	0.6
		SE	0.3	5.8	5	0.5	7	0.04	0.1	0.05	----	0.1	0.7	1.2	0.3
		n	5	5	5	5	5	5	4	4	1	4	4	5	4
4	A	334	93.6	84.5	80	1.0	---	6.90	----	----	----	5.1	58.7	35.0	20.5
		377	89.0	84.3	40	0.4	340	6.97	----	----	----	4.7	58.8	36.1	21.2
		395	94.4	84.0	40	0.7	317	----	----	----	----	4.4	53.7	36.2	19.4
		1825	96.6	89.1	34	0.3	---	7.03	17.1	0.35	----	3.5	41.0	37.5	15.4
		1893	93.5	----	48	0.9	301	6.99	15.4	0.25	27.5	3.7	45.0	35.8	17.8
		1893	96.6	87.6	41	0.6	296	7.02	15.5	0.35	26.3	3.7	45.0	36.0	16.3
		1893	97.7	80.4	21	0.4	310	7.01	17.3	0.35	27.9	4.1	45.0	36.7	16.6
		1893	95.6	88.3	34	0.3	313	7.04	17.5	0.40	26.7	4.2	51.0	35.9	16.4
		Mean	94.6	85.5	42	0.6	313	6.99	16.6	0.34	27.1	4.2	49.8	36.2	18.0
		SD	2.7	3.1	17	0.3	15	0.05	1.0	0.06	0.7	0.5	6.8	0.7	2.2
		SE	1.0	1.2	6	0.1	6	0.02	0.5	0.02	0.4	0.2	2.4	0.3	0.8
		n	8	7	8	8	6	7	5	5	4	8	8	8	8

Horse No.	Bleed	Days Fzn (-150 C)	Recovery (%) Fz-Thaw	Recovery (%) Freeze-Thaw-Wash	Supt. Hb (mg%)	Extra K^+ (mEq/l)	Supt. Osmolal. (mOsm/kg H_2O)	Blood pH	RBC 2,3 DPG (uM/g Hb)	RBC ATP (uM/g Hb)	RBC P_{50} (mm Hg)	RBC K^+ (mEq/10^{12} RBC)	MCV (u^3)	MCHC (%)	MCH (uug)
4	B	80	96.4	89.5	75	0.5	311	6.96	12.0	0.31	30.7	4.4	50.0	38.6	19.3
		332	96.3	86.8	40	0.4	---	6.96	----	----	----	4.7	59.1	36.2	21.4
		392	96.7	89.4	20	1.0	317	----	----	----	----	4.5	53.8	35.4	19.0
		1305	96.0	87.2	88	0.4	373	----	13.0	0.68	27.2	4.9	54.2	38.0	20.8
		1890	97.7	89.6	14	0.4	297	7.07	18.0	0.30	23.0	4.0	46.0	35.2	16.3
		Mean	96.6	88.5	47	0.5	325	7.00	14.3	0.43	27.0	4.5	52.6	36.7	19.4
		SD	0.6	1.4	33	0.3	33	0.06	3.2	0.22	3.9	0.4	4.9	1.5	2.0
		SE	0.3	0.6	15	0.1	17	0.04	1.9	0.13	2.2	0.2	2.2	0.7	0.9
		n	5	5	5	5	4	3	3	3	3	5	5	5	5
5	A	86	95.2	80.3	70	0.6	314	6.96	14.7	0.27	28.8	3.6	40.3	37.2	15.0
		232	97.0	86.4	90	0.3	340	6.97	----	----	----	---	----	----	----
		1821	97.9	89.2	28	0.3	---	7.07	----	0.35	----	5.2	58.0	40.0	21.8
		Mean	96.7	85.3	63	0.4	327	7.00	14.7	0.31	28.8	4.4	49.2	38.6	18.4
		SD	1.4	4.6	32	0.2	18	0.06	----	0.06	----	1.1	12.6	2.0	4.8
		SE	0.8	2.6	18	0.1	13	0.04	----	0.04	----	0.8	8.9	1.4	3.4
		n	3	3	3	3	2	3	1	2	1	2	2	2	2
5	B	6	95.6	90.2	100	0.4	391	7.13	----	----	----	---	----	38.7	----
		371	95.4	83.5	70	1.4	339	6.99	----	----	----	---	----	36.0	----
		Mean	95.5	86.9	85	0.9	365	7.06	----	----	----	---	----	37.4	----
		SD	0.1	4.7	21	0.7	37	0.10	----	----	----	---	----	1.9	----
		SE	0.1	3.4	15	0.5	26	0.07	----	----	----	---	----	1.4	----
		n	2	2	2	2	2	2	----	----	----	---	----	2	----
Mean of 9 Studies		Mean	96.2	85.2	52	0.9	323	7.06	14.8	0.31	28.1	4.1	47.9	36.4	17.5
		SD	0.7	3.1	18	0.4	18	0.10	0.9	0.10	1.3	0.5	4.1	0.6	2.0
		SE	0.2	1.0	6	0.1	6	0.03	0.3	0.03	0.5	0.2	1.5	1.6	0.7
		n	9	9	9	9	9	9	8	8	7	7	7	8	7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